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Previous Nutritional State and Glucose Modulate Glucagon-Mediated Hepatic Lipolysis in Rainbow Trout, *Oncorhynchus mykiss*

Jamie S. Harmon and Mark A. Sheridan[1]

Department of Zoology, North Dakota State University
Fargo, ND 58105, U.S.A.

ABSTRACT—Rainbow trout were used to investigate the effects of previous nutritional state and glucose presence on glucagon-stimulated hepatic lipolysis. Experiments were conducted *in vitro* on liver removed from fed fish and from fish fasted for 4 or 6 weeks. Basal hepatic lipolysis was enhanced in liver removed from fasted fish compared to fed animals. Glucagon-stimulated lipolysis was more pronounced in liver removed from 4-week fasted fish than in liver isolated from fed fish. Glucagon failed to affect hepatic lipolysis in the liver of 6-week fasted animals. The effects of glucose presence and absence were also examined. Basal lipolysis in liver tissue incubated in the presence of glucose was more pronounced than rates observed in tissue cultured in the absence of glucose. Glucagon stimulated lipolysis in liver incubated in the presence of glucose to a greater extent than liver incubated in the absence of glucose. Insulin inhibited glucagon-stimulated lipolysis in liver from fed and fasted fish as well as in liver cultured in the presence and absence of glucose. These results indicate that glucagon-mediated lipolysis is modulated by previous nutritional state and by glucose.

INTRODUCTION

Hepatic lipolysis in trout is mediated by a triacylglycerol lipase enzyme which hydrolyzes stored triacylglycerol (TG) to glycerol and fatty acids (FA) [1]. A number of hormones have been found to influence lipolysis in fish [2] and recently we have indicated a role of glucagon family peptide (glucagon, glucagon-like peptide)-mediated hepatic lipolysis [3]. *In vivo* administration of glucagon (GLU) or glucagon-like peptide (GLP) resulted in elevated plasma FA accompanied by enhanced hepatic TG lipase activity [3]. Recently, we have demonstrated that GLU acts directly on hepatic lipolysis. In rainbow trout liver incubated *in vitro*, glucagon stimulated tissue TG lipase activity as well as FA and glycerol release into culture medium [4].

Previous nutritional state has also been shown to profoundly influence the intermediary metabolism of fish. Coho salmon (*Oncorhynchus kisutch*) fasted for 3 weeks displayed elevated plasma FA concentrations accompanied by enhanced hepatic TG lipase activity [5]. These fish also exhibited reduced titers of insulin (INS), GLU and GLP compared to their fed counterparts. A similar observation was found in rainbow trout fasted for 6 weeks [6]. Hepatic lipolytic activity in liver removed from fasted (4 weeks) trout and cultured *in vitro* displayed enhanced activity over liver removed from fed animals and similarly cultured [4]. Glucose also influences hepatic lipolysis while altering the pattern of pancreatic hormones (insulin, glucagon, glucagon-like peptide and somatostatin-25). Glucose injection into rainbow trout resulted in elevated plasma FA levels attended by enhanced hepatic lipolysis [7]. The extent to which nutritional state and/or glucose interacts with hormones to modulate lipid mobilization in fish is not known.

In the present study, we used rainbow trout to examine the effects of previous nutritional state and glucose presence on glucagon-stimulated hepatic lipolysis. The specific questions we addressed were: 1) What are the effects of glucagon on lipolysis in liver removed from animals of varying nutritional states? and 2) What are the effects of glucagon on lipolysis in liver removed from fed fish and incubated in the presence or

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[1] To whom all correspondence should be addressed.

absence of glucose? We also report the effects of insulin on lipolysis in liver removed from animals of varying nutritional states and in liver incubated in the presence or absence of glucose.

MATERIALS AND METHODS

Experimental animals

Yearling rainbow trout (mean weight ± SEM, 54 ± 5 g) of both sexes were obtained from Garrison National Fish Hatchery near Riverdale, ND and maintained at North Dakota State University in dechlorinated municipal water (13°C) under 12L : 12D photoperiod. Fish were fed *ad libitum* twice daily with Glencoe Mills Trout Chow (Glencoe, MN).

Experiments were conducted in the fall of the year. Animals were divided into three groups of varying nutritional regimes: fed continuously (except 24–36 hr prior to experimentation), fasted 4 weeks, and fasted 6 weeks. Immediately prior to experimentation, fish were anesthetized in buffered 0.01% (w/v) tricaine methanesulfonate (MS-222), bled from the severed caudal vessels, and the livers removed and prepared for organ culture.

Organ culture and incubation conditions

In vitro experiments were carried out on livers removed from several lots of fish (10–12 individuals per lot) within each of the nutritional groups. Livers were carefully perfused by means of syringe and needle with cold (14°C) saline (0.75%, w/v) until cleared of blood. Livers were delicately cut into ca. 1-mm^3 pieces [8], placed into 35-ml plastic centrifuge tubes containing glucose-free or glucose-containing Hanks solution (137 mM NaCl, 5.4 mM KCl, 4.0 mM $NaHCO_3$, 1.7 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 10 mM HEPES, pH 7.6, containing 0.24% (w/v) bovine serum albumin and 5.55 mM glucose, the normal level of plasma glucose), and preincubated in darkness with a gyratory shaker (150 rpm) under 100% O_2 at 14°C to stabilize tissue pieces. After 15 min, the pieces were centrifuged (270×*g* for 5 min at 14°C) and washed three times by resuspension-centrifugation. For nutritional state experiments, liver pieces (4–6 pieces per well, ca. 15 mg fresh weight; representing ca. 70 μg protein/mg fresh weight) from animals of each nutritional regime were transferred to each well of 24-well plastic culture plates (Falcon 3047) containing 1 ml (per well) of glucose-containing Hanks medium, into which hormone was previously dissolved. Hormone treatments consisted of either GLU (bovine/porcine, Sigma G4250), INS (bovine, Sigma I5500), or a combination of GLU and INS. Hormones were added to a final concentration of 10^{-6} M; this concentration was found to be maximally effective at stimulating trout hepatic lipolysis [4]. Mammalian hormones were chosen because of the scarcity of salmonid peptides and because of the similarity in function between mammalian and salmon hormones in salmonid systems [cf. 3]. In hormone combination treatments, INS was added alone for 5 min prior to addition of INS and GLU together; addition of INS plus GLU-containing medium initiated the incubation (time = 0). Incubation (under gyration at 100 rpm) proceeded for a period of 5 hr under 100% O_2 at 14°C. Preliminary studies indicated that maximum effects were observed at 5 hr of incubation. For glucose experiments, liver pieces (4 to 6 pieces, ca. 15 mg fresh weight) from continuously fed animals were transferred to each well of multi-well plates containing 1 ml (per well) of Hanks glucose-containing or glucose-free medium. Treatments (control, single and combination hormone additions) and incubation were conducted as described for nutritional state experiments. In all experiments, tissue and medium samples were frozen and stored at −90°C for later analyses (usually within two weeks).

Biochemical analyses

Prior to fatty acid and glycerol analysis, samples were deproteinated by heat treatment (65°C for 10 min) followed by centrifugation (16,000×*g* for 10 min). Medium fatty acid (FA) levels were measured by the micromethod on Noma et al. [9]. Glycerol release was measured by the method of Worthington [10]. Protein was determined by the dye-binding method [11] using a Bio-Rad (Richmond, CA) microplate reader (cf., Bio-Rad Technical Bulletin 1177). Lipolytic capacity was assessed by measuring the triacylglycerol (TG) lipase activity in partially purified preparations as described by Sheridan et al. [12].

Statistics

Results are expressed as means ± SEM. Statistical differences were estimated by analysis of variance; multiple comparisons among means were made by the Student-Newman-Keuls test; alpha was set at 0.05.

RESULTS

Effects of nutritional state on hepatic lipolysis

The effects of nutritional state on hepatic lipolysis were assessed by measuring the (TG) lipase activity of liver removed from fed and fasted (4 and 6 weeks) fish and cultured for 5 hr (Fig. 1). Liver removed from fish that were fasted showed a significant ($P<0.05$) increase in lipolytic activity as compared to that observed in liver removed from fed animals. Basal hepatic lipolysis in liver from 6-week fasted fish was not as pronounced as that in liver from 4-week fasted animals.

Glucagon stimulated a significant ($P<0.05$) increase in lipase activity in liver isolated from fed fish and in liver removed from fish fasted for 4 weeks. Glucagon-stimulated lipolysis was more pronounced in liver removed from 4-week fasted fish than in liver removed from fed animals (Fig. 1). Glucagon failed to affect hepatic lipolysis in liver isolated from 6-week fasted animals.

The presence of INS within the culture medium caused a decrease ($P<0.05$) in hepatic lipase activity in the 4-week fasted fish, whereas there appeared to be no affect of INS on liver removed from other groups (Fig. 1).

With the combination of INS plus GLU, INS inhibited glucagon-stimulated lipolysis in liver isolated from fed fish as well as from fish fasted 4 weeks (Fig. 1).

Effects of glucose on hepatic lipolysis

The effects of glucose presence on hepatic lipolysis were determined in liver removed from fed fish and cultured for 5 hr (Table 1). Basal

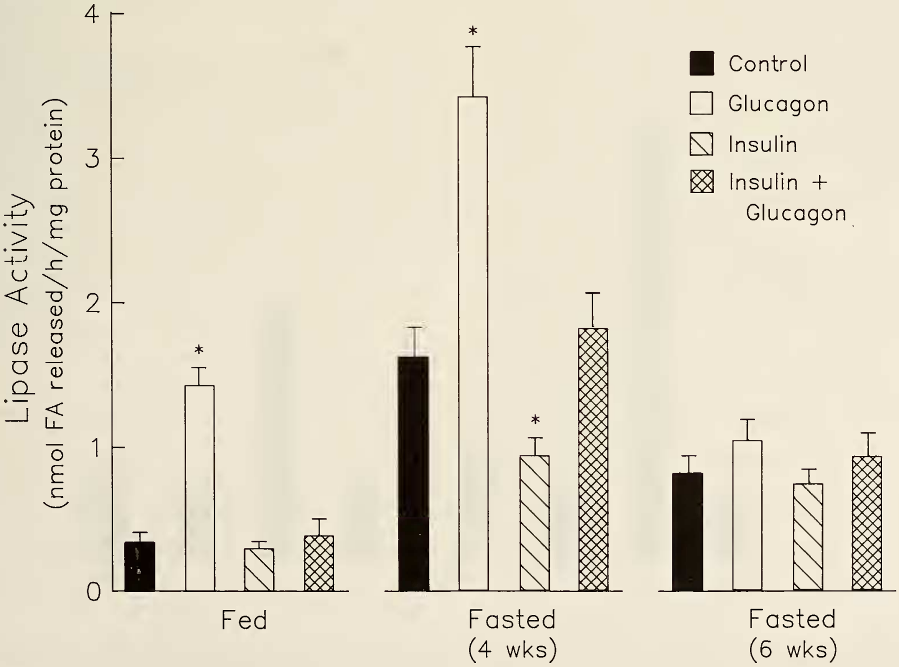

FIG. 1. Effects of glucagon and insulin on triacylglycerol lipase activity in liver removed from rainbow trout of varying nutritional states and cultured *in vitro* for 5 hr in glucose-containing (5.5 mM) medium. Liver pieces were cultured in medium containing no hormones, insulin (10^{-6} M), glucagon (10^{-6} M), or insulin (10^{-6} M) plus glucagon (10^{-6} M). Data presented as means ± SEM (n=12). * Significantly different from control, $P<0.05$.

hepatic lipolysis, as assessed by measuring TG lipase activity, was enhanced ($P<0.05$) in liver incubated in glucose-containing medium over that in liver cultured in glucose-deficient medium. Glucagon significantly stimulated ($P<0.05$) hepatic lipolysis. Notably, glucagon-stimulated lipolysis was more pronounced in liver incubated in the presence of glucose (Table 1). Insulin alone appeared to have no affect on basal hepatic lipolysis. In combination, INS inhibited glucagon-stimulated lipolysis in liver incubated both in the presence and absence of glucose (Table 1).

Lipolysis was also reflected in the concentrations of medium metabolites. Liver was removed from fed fish and incubated for 5 hr in glucose-containing or glucose-deficient medium, and the concentrations of FA and glycerol were determined (Fig. 2). Glucagon stimulated a significant increase ($P<0.05$) in FA and glycerol release. This increase was further enhanced by the presence of glucose. The presence of INS or INS plus GLU indicated no significant differences in FA and glycerol release between the glucose-containing and glucose-deficient medium.

TABLE 1. Effects of glucagon and insulin on triacylglycerol lipase activity in rainbow trout liver[a]

Treatment	Glucose +	Glucose −
Control	0.41±0.07	0.24±0.05
Glucagon	1.98±0.08[b]	0.84±0.11[c]
Insulin	0.32±0.04	0.28±0.06
Insulin+Glucagon	0.46±0.06	0.34±0.09

[a] Liver tissue removed from fed fish and cultured in modified Hanks medium for 5 hr. Enzyme activity expressed as nmol fatty acid released/h/mg protein. Data presented as means±SEM (n=12).

[b,c] Significantly different from respective control ($P<0.05$); b significantly different from c ($P<0.05$).

DISCUSSION

The results of the present study indicate that previous nutritional state and glucose presence

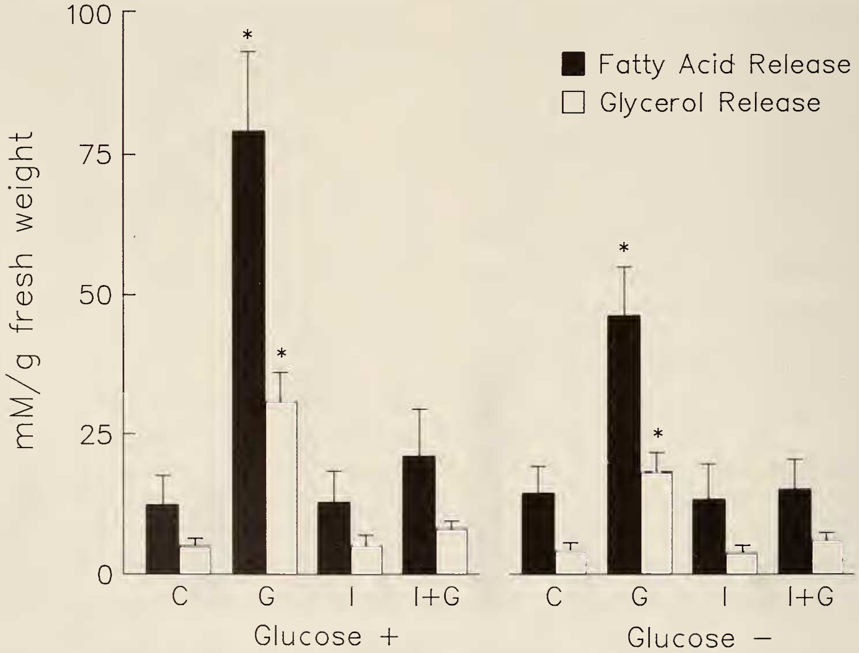

FIG. 2. Effects of glucagon and insulin on fatty acid and glycerol release from rainbow trout liver removed from fed fish and cultured *in vitro* in the presence or absence of glucose (5.5 mM) for 5 hr. Liver pieces were cultured in medium containing no hormones (control, C), 10^{-6} M insulin (I), 10^{-6} M glucagon (G), or 10^{-6} M insulin plus 10^{-6} M glucagon (I+G). Data presented as means±SEM (n=12). * Significantly different from control, $P<0.05$.

influence both basal and GLU-mediated hepatic lipolysis in rainbow trout.

Fasting resulted in elevated hepatic lipolytic activity. This observation is consistent with our previous finding that hepatic lipolysis is more pronounced in liver removed from fish fasted for 4 weeks than in liver removed from fed fish [4]. In our present experiments, we found for the first time that an extended fast (6 weeks) did not result in lipolytic rates as high as those seen in tissue from shorter-term (4-week) fasted animals. This may result from exhaustion of lipid reserves during fasting. Lipids have been shown to be depleted from coho salmon liver during a 4-week fast [5] and during smoltification and seawater adaptation [13]. Alterations in lipase activity during fasting of coho salmon were correlated with decreases in INS/GLU and INS/GLP ratios in plasma [5]. Fasting-associated alterations in the profile of pancreatic hormones were also reported in trout [6]. Such hormonal patterns may influence the activation state of the lipase enzyme. Schwartz and Jungas [14] suggested that fasting-associated lipolysis results from inhibition of the lipase inactivating system.

Glucagon stimulated lipolytic activity in liver removed from fed fish and from fish fasted 4 weeks. While GLU is known to promote lipolysis in fed fish [4], we report for the first time that GLU-stimulated lipolysis is more pronounced in tissue removed from 4-week fasted fish than in tissue removed from fed animals. Fasting has also been reported to sensitize adipocytes to norepinephrine-stimulated lipolysis in rats [15]. Notably, GLU failed to enhance hepatic lipolysis in trout liver removed from animals fasted for 6 weeks. The basis of this observation is not known, although it may result from the already advanced state of lipid depletion. Similar observations were made with hepatic glycogenolysis in chinook salmon [8]. In these experiments, GLU stimulated glycogenolysis in liver from fed animals but failed to promote glycogenolysis in liver from longer-term fasted fish. Other factors may also influence reduced GLU sensitivity. "Refractoriness" to sequential lipolytic stimulus has been reported in rat adipose tissue [16] and in various tissues of salmon after smoltification [13]. During fasting, the relative abundance of GLU in the plasma of fish was higher than in fed animals [5, 6]; these altered hormonal patterns may alter receptor characteristics.

In our present experiments we observed that glucose appeared to stimulate hepatic lipolysis. This observation is consistent with our previous findings that basal hepatic lipolysis is enhanced in the presence of glucose [4]. Glucose has been reported to increase basal lipolysis in adipose tissue isolated from mammals as well [17]. It is interesting to note that lipolysis in trout liver incubated in the presence of glucose results in the release of FA and glycerol in less than the expected 3:1 ratio. This suggests that while lipolysis is proceeding at higher rates, the extent of reesterification is also elevated. A similar situation was observed in mammalian adipose tissue [17]. *In vivo* administration of glucose to trout results in elevated plasma FA supported by increased hepatic lipolysis [7]. These metabolic alterations were associated with reduced plasma levels of somatostatin and GLU—a pattern similar to that seen in diabetic humans [18]. In normal humans, glucose infusions lowered plasma FA via insulin-stimulated uptake [19] and resulted in increased rates of FA reesterification and reduced lipid mobilization from adipose tissue [20]. Wolfe and Peters [20] concluded, however, that glucose increased TG-FA cycling—a contention supported by our present experiments.

We also report for the first time that GLU stimulates lipolysis in liver incubated in the presence of glucose to a greater extent than liver incubated in the absence of glucose. This is evidenced by alterations in tissue lipase activity as well as by increased medium FA and glycerol release. Hormone-stimulated lipolysis, as indicated by glycerol release, also appeared to be enhanced in epinephrine- [17] and norepinephrine-treated [21] adipose tissue isolated from the rat.

Insulin inhibited glucagon-stimulated lipolysis in liver from fed fish and in liver from fish fasted for 4 weeks. Insulin appears to exert a net antilipolytic action in fish fasted for 4 weeks. The antilipolytic action of INS in mammals is well known [18]. We have also previously reported an antilipolytic action of INS in liver removed from fed trout [4].

The relationship between the antilipolytic action of INS and nutritional state is complex and has not been well addressed in fish. Emdin [22] reported that INS injection did not result in a difference in lipid metabolism between fed and fasted hagfish.

Insulin also inhibited glucagon-stimulated lipolysis in trout liver incubated in the presence and absence of glucose. The inhibitory action of INS on glucagon-stimulated lipolysis in the presence of glucose confirms our previous observations on INS action [4]. While glucose presence enhanced FA reesterification in trout liver, INS appeared not to appreciably affect this process. In rat adipose tissue, however, INS strongly increased the level of reesterification [17]. These authors also have shown that the combined effects on INS and epinephrine in the presence of glucose resulted in substantially more reesterification than in the absence of glucose [17]. Dominguez and Herrera [23] found that INS altered glycerol utilization in adipose tissue and suggested that these effects were altered by the presence of glucose. The precise manner by which INS affects lipolysis and reesterification remains to be determined.

In summary, the results of this study advance our fundamental understanding of the control of lipid metabolism in fish in two ways: 1) the lipolytic action of GLU is enhanced during shorter-term fasting, and 2) the lipolytic action of GLU is enhanced in the presence of glucose. The role of GLU in regulating lipid metabolism in fish has been somewhat confusing, since some reports indicate that GLU has lipolytic effects while other reports maintain that GLU has no lipolytic action [2]. Recently, Sheridan suggested that the basis of this conflict may lie in differences in fish life history patterns [24]. This contention is supported by the report that the *in vivo* effects of GLU in salmon were seasonally dependent [3]. The present findings suggest that nutritional state and glucose charge, underlied by hormonal status, also must be considered when evaluating GLU action in fish.

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